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Differential accumulation of genetic and phenotypic changes in Venezuelan equine encephalitis virus and Japanese encephalitis virus following passage *in vitro* and *in vivo*

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ABSTRACT

The requirement to replicate in both vertebrate and invertebrate hosts is thought to limit the introduction of genetic changes into the genome of arboviruses. Serial passage under laboratory conditions will overcome this limitation allowing for genetic changes to be introduced and affecting the virulence of the virus for animals. In the studies detailed here, the consequence of removing the restriction of alternate replication was demonstrated to be different depending on the virus. Passing Venezuelan equine encephalitis virus in tissue culture cells, eggs or mice resulted in up to 11 nucleotide or amino acid changes but no significant change in the virulence of the virus for mice. Passing Japanese encephalitis virus (JEV) under the identical conditions resulted in as many as 22 nucleotide or amino acid changes that often resulted in improved survival probabilities. For JEV, most genetic changes along with the attenuated phenotype were selected within 5 passes.

Introduction

Venezuelan equine encephalitis virus (VEEV, genus *Alphavirus*, family *Togaviridae*) and Japanese encephalitis virus (JEV, genus *Flavivirus*, family *Flaviviridae*) share common characteristics including a positive-sense, single-stranded RNA genome; an enveloped icosahedral nucleocapsid; and transmission between susceptible vertebrate hosts by arthropod vectors (Geiss et al., 2009; Gould et al., 2009; Jose et al., 2009). In general, RNA viruses have the capacity for significant genetic variation due to the error prone nature of the viral RNA-dependent RNA polymerase which is responsible for replication of the viral genome but has no proofreading activities (Drake and Holland, 1999; Duarte et al., 1994; Steinhauer et al., 1992). Paradoxically, the genomic sequence of arthropod-borne RNA viruses, such as those in the alphavirus and flavivirus genera, remain stable over long periods of time when compared to viruses transmitted directly between susceptible hosts, likely due to the requirement for alternate replication in vertebrate and invertebrate hosts (Jenkins et al., 2002; Vasilakis et al., 2009).

Although humans do not participate in the transmission cycle of VEEV and JEV, humans are susceptible to infection, often with significant morbidity and mortality (Calisher, 1994; Erlanger et al., 2009; Mackenzie et al., 2004; Zacks and Paessler, 2010). Most people infected with enzootic strains of VEEV (subtypes IAB and IC) will develop an acute, febrile disease, which may be incapacitating and have a prolonged convalescence period. Approximately 14% of cases will progress to neurological involvement and encephalitis with a 1% fatality rate (Franz et al., 2001; Zacks and Paessler, 2010). VEEV is also highly infectious by the aerosol route and has been associated with numerous laboratory acquired infections (Bellamy and Freedman, 2001; Kadlec et al., 1997; Zacks and Paessler, 2010). JEV is the leading cause of viral encephalitis in Asia and nearly half of the current human population lives in JEV endemic areas (Erlanger et al., 2009; Lowry et al., 1998). The annual incidence of Japanese encephalitis ranges from 30,000 to 50,000 cases, with mortality estimated to be between 25% and 30%. A third of survivors are left with significant neurologic sequelae including memory loss, motor weakness and behavioral disturbances. VEEV and JEV are both listed as priority pathogens by the National Institute of Allergy and Infectious Diseases because of their potential to be used as biological weapons.

The theory of constrained evolution as a result of alternating hosts is supported by numerous studies in which alphaviruses or flaviviruses

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were passed sequentially within or between vertebrate and invertebrate hosts (Ciota et al., 2009; Coffey et al., 2008; Greene et al., 2005; Jerzak et al., 2007; Jerzak et al., 2008; Vasilakis et al., 2009; Weaver et al., 1999). Generally speaking, passing a virus sequentially in a specific host type resulted in the introduction of genetic changes and corresponding fitness gains for that host, whilst diminishing fitness in the alternate host. Alternately passing an alphavirus or a flavivirus between vertebrate and invertebrate hosts, as occurs in nature, resulted in fewer genetic substitutions and either no change in the fitness of the virus or increased fitness for both hosts. The number and rate at which genetic changes accumulate in or between alphaviruses or flaviviruses following sequential passage *in vitro* and *in vivo* were not compared in these studies. Additionally, the effect of serial passage on the phenotype of the virus in an animal model was often not assessed.

Live attenuated vaccines for many different viruses, including VEEV and JEV, have been developed by repeated *in vitro* passage (Ada, 2001; Kinney et al., 1993; Ni et al., 1995; Nitayaphan et al., 1990). The methods used to perform these passages were different for VEEV and JEV; however, it is notable that there were a minimum of 45 nucleotide changes and 15 amino acid changes introduced into the JEV genome following passage to select for an attenuated virus (Nitayaphan et al., 1990) as compared to 12 nucleotide and 8 amino acid changes introduced following passage of VEEV (Kinney et al., 1993). The effect of alternating passage of VEEV and JEV between vertebrate and invertebrate cells on the virulence of the virus has not been assessed, however if this method serves to constrain the introduction of genetic changes in the virus, then it would be reasonable to assume that passing the virus in this way would also conserve the virulence of the virus.

Repeated passage of some viruses *in vivo* has also been used to develop live attenuated vaccines in the past (Ada, 2001); however, for Sindbis virus (SINV), an alphavirus, and St. Louis encephalitis virus (SLEV), a flavivirus, repeated passage in animals selected for genetic variants with increased infectivity for that animal (Ciota et al., 2009; Lustig et al., 1992). For SINV, passage in mouse brains resulted in a virus that demonstrated increased neurovirulence and neuroinfectivity in mice when compared to the original virus. Similarly, passing SLEV in chickens resulted in a virus with increased infectivity for chickens; however, the pathogenicity of SLEV in comparison to the parent was not assessed and increased infectivity was not observed in all chicken passage lineages. These conflicting results suggest that the effect of passing viruses in animals is uncertain and may depend on the virus and the passage conditions.

Tissue culture cells, suckling mice and eggs each represent vehicles that can be used to propagate VEEV and JEV. Based on the results of previous studies summarized above, it would be predicted that passage of these viruses in or between mammalian and mosquito tissue culture cells would result in the accumulation of genetic changes and viral attenuation. The effect of passing VEEV and JEV in eggs or mice is less certain but would be expected to at least retain the virulence properties of the viruses. In order to test these predictions VEEV and JEV were passed up to 40 times in or between vertebrate and invertebrate tissue culture cells, and in suckling mice and eggs a total of 20 times. The genomic sequence of the resulting viruses and their virulence in weanling CD-1 mice were compared.

Results

Accumulation of genetic changes following passage of VEEV

The genetic changes observed following passage of VEEV in Vero (African green monkey kidney) cells; C6/36 (*Aedes albopictus* mosquito) cells; alternately between Vero and C6/36 cells (Alt); in suckling mice (MB); or in eggs are summarized in Table 1. The VEEV Trinidad Donkey (VEEV TRD) strain was used to initiate each of the passage stocks and serves as the reference strain for all VEEV comparisons. VEEV TRD is commonly used in laboratory experiments to represent fully virulent VEEV; however the passage history prior to initiating these studies is unknown and the implications on the results are discussed.

Table 1
Nucleotide and amino acid changes in VEEV following passage.

Region[a]	Position[b]	Virus stock					
		VEEV TRD	VEEV Vero	VEEV C6/36	VEEV Alt	VEEV Egg	VEEV MB
5′ NCR	–	–	–	–	–	–	–
nsP1	–	–	–	–	–	–	–
nsP2	aa466	Pro	–	Pro/Leu	–	–	–
nsP3	aa288	Gln	–	–	Arg	–	–
	aa471	His	His/Arg	–	–	–	–
	aa478	Thr	–	Thr/Ile	–	–	–
nsP4	aa207	Val	–	Val/Ile	–	–	–
	aa451	Glu	–	Glu/Lys	–	–	–
C	aa54	Asp	–	Asp/Asn	–	–	–
E3	–	–	–	–	–	–	–
E2	aa56	Asp	–	–	–	Gly	–
	aa120	Lys/Thr[c]	Lys	Lys	Lys	Thr	Thr
	aa158	Ala	–	–	–	Glu	–
	aa159	Gln	Gln/Pro	–	–	–	–
	aa165	Val	–	Val/Ala	–	–	–
	aa168	His	–	His/Arg	–	–	–
	aa205	Thr	Lys	Thr/Lys	–	–	Lys
	aa211	Asn	–	Asn/Tyr	–	–	–
6K protein	aa51	Ala	–	Ala/Thr	–	–	–
E1	aa248	Pro	–	–	Thr	–	–
3′ NCR	nt11393	U	–	–	–	A	–

–, no change.
[a] Non-coding region (NCR) or viral protein.
[b] Nucleotide (nt) or amino acid (aa) position within the viral genome or within a given gene.
[c] Mixed population.

Sequencing of the VEEV TRD stocks indicated a mixed population of Lys or Thr at amino acid 120 of the E2 glycoprotein (E2 120). Mixed populations were identified as multiple peaks within the chromatogram at a given nucleotide position. Because consensus sequencing was used for these experiments single peaks indicate that a given nucleotide was present in the majority population. Passing VEEV TRD in, or alternating between Vero and C6/36 cells, up to 40 times selected for Lys at this position; while passing in eggs or suckling mice up to 20 times selected for Thr. VEEV Egg had two additional amino acid changes in the E2 glycoprotein plus a nucleotide change in the 3′ non-coding region (NCR) that were not introduced until after 15 passes (data not shown). VEEV MB had only one additional amino acid change in the E2 glycoprotein.

It took between 15 and 20 passes for Lys to become predominant in the majority population at E2 120 of both VEEV Vero and VEEV Alt (Table 2). Two additional amino acid changes, one in nonstructural protein 3 (nsP3) and one in the E1 glycoprotein were introduced into VEEV Alt, between passes 10 and 15, and became predominant in the majority population between pass 20 and 25. Three additional changes were present in VEEV Vero after 40 passes including mixed populations at E2 159 and nsP3 471 (Table 1). These genetic changes were introduced after 10 or 15 passes, respectively (Table 2). The amino acid change at E2 205 of VEEV Vero was introduced sometime after the 5th pass and became predominant in the majority population sometime between 20 and 25 passes. VEEV C6/36 contained a mixed population with 10 variable sites located in the nsP2, nsP3, nsP4, Capsid (C), E2 and 6K proteins after 40 passes (Table 1). It took more than 30 passes for these variable sites to be introduced, yet the genetic change at E2 120 became predominant in the majority population after 10 passes (Table 2).

Effect of passage on the virulence of VEEV

The percent mortality for VEEV stocks is calculated as the total number of mice that died as a result of virus challenge versus the total number of mice challenged and is presented in Fig. 1. VEEV TRD

Table 2
Accumulation of amino acid changes in VEEV following passage in tissue culture cells.

Region[a]	Position[b]	Virus stock								
		VEEV TRD	VEEV Vero P5	VEEV Vero P10	VEEV Vero P15	VEEV Vero P20	VEEV Vero P25	VEEV Vero P30	VEEV Vero P35	VEEV Vero P40
nsP1	–	–	–	–	–	–	–	–	–	–
nsP2	–	–	–	–	–	–	–	–	–	–
nsP3	aa471	His	–	–	–	His/Arg	His/Arg	His/Arg	His/Arg	His/Arg
nsP4	–	–	–	–	–	–	–	–	–	–
C	–	–	–	–	–	–	–	–	–	–
E3	–	–	–	–	–	–	–	–	–	–
E2	aa120	Lys/Thr	Lys	Lys/Thr	Lys/Thr	Lys	Lys	Lys	Lys	Lys
	aa159	Gln	–	–	Gln/Pro	Gln/Pro	Gln/Pro	Gln/Pro	Gln/Pro	Gln/Pro
	aa205	Thr	–	Thr/Lys	Thr/Lys	Thr/Lys	Lys	Lys	Lys	Lys
6K	–	–	–	–	–	–	–	–	–	–
E1	–	–	–	–	–	–	–	–	–	–

Region	Position	Virus stock								
		VEEV TRD	VEEV C6/36 P5	VEEV C6/36 P10	VEEV C6/36 P15	VEEV C6/36 P20	VEEV C6/36 P25	VEEV C6/36 P30	VEEV C6/36 P35	VEEV C6/36 P40
nsP1	–	–	–	–	–	–	–	–	–	–
nsP2	aa466	Pro	–	–	–	–	–	–	Pro/Leu	Pro/Leu
nsP3	aa478	Thr	–	–	–	–	–	–	Thr/Ile	Thr/Ile
nsP4	aa207	Val	–	–	–	–	–	–	Val/Ile	Val/Ile
	aa451	Glu	–	–	–	–	–	–	Glu/Lys	Glu/Lys
C	aa54	Asp	–	–	–	–	–	–	Asp/Asn	Asp/Asn
E3	–	–	–	–	–	–	–	–	–	–
E2	aa120	Lys/Thr	Lys	Lys/Thr	Lys	Lys	Lys	Lys	Lys	Lys
	aa165	Val	–	–	–	–	–	–	Val/Ala	Val/Ala
	aa168	His	–	–	–	–	–	–	–	His/Arg
	aa205	Thr	Thr/Lys	Thr	Thr	Thr	Lys	Lys	Lys	Thr/Lys
	aa211	Asn	–	–	–	–	–	–	–	Asn/Tyr
6K	aa51	Ala	–	–	–	–	–	–	Ala/Thr	Ala/Thr
E1	–	–	–	–	–	–	–	–	–	–

Region[a]	Position[b]	Virus stock								
		VEEV TRD	VEEV Alt P5	VEEV Alt P10	VEEV Alt P15	VEEV Alt P20	VEEV Alt P25	VEEV Alt P30	VEEV Alt P35	VEEV Alt P40
nsP1	–	–	–	–	–	–	–	–	–	–
nsP2	–	–	–	–	–	–	–	–	–	–
nsP3	aa288	Gln	–	–	Gln/Arg	Gln/Arg	Arg	Arg	Arg	Arg
nsP4	–	–	–	–	–	–	–	–	–	–
C	–	–	–	–	–	–	–	–	–	–
E3	–	–	–	–	–	–	–	–	–	–
E2	aa120	Lys/Thr[c]	–	–	–	Lys	Lys	Lys	Lys	Lys
6K	–	–	–	–	–	–	–	–	–	–
E1	aa248	Pro	–	–	Pro/Thr	Pro/Thr	Thr	Thr	Thr	Thr

–, no change.
[a] Viral protein.
[b] Amino acid (aa) position within a given gene.
[c] Mixed population.

resulted in 100% mortality when administered via the subcutaneous (SC) route and 60% mortality via the intranasal (IN) route. When mice were challenged with VEEV Vero, VEEV C6/36, VEEV Alt, VEEV MB and VEEV Egg via the IN route, no significant differences were observed in the percent mortality when compared to VEEV TRD (Fig. 1). With the exception of VEEV Vero ($P=0.022$) survival probabilities, following IN exposure were also not significantly affected (Fig. 2). Survival probabilities focus on the time to death as well as the probability of death; thus for VEEV Vero the survival curve was statistically different when compared to VEEV TRD even though the percent mortality did not change significantly. Following SC exposure, the percent mortality was decreased when mice were challenged with VEEV C6/36 only ($P=0.033$); however survival probabilities improved when mice were challenged with VEEV Vero ($P=0.001$), VEEV Alt ($P<0.001$) and VEEV Egg ($P=0.028$) in addition to VEEV C6/36 ($P=0.003$). The survival probability for mice challenged with VEEV MB decreased ($P=0.001$) in comparison to VEEV TRD following SC exposure though the percent mortality was unchanged. Virulence studies conducted using earlier passes of VEEV with unique genetic changes did not provide any significant data that would help to determine the role of specific genetic changes on the virus phenotype (data not shown).

Accumulation of genetic changes following passage of JEV

Based on whole genome sequencing, the stock of JEV Nakayama (JEV Nak) which was used to initiate passage studies for JEV contained mixed populations of Lys or Arg at amino acid 209 of the Envelope (E) protein and amino acid 8 of non-structural protein 4b (NS4b); Ile or Val at amino acid 405 of the NS5 protein; cytidine (C) or uracil (U) at nucleotide 10431 of the 3′ NCR; and adenosine (A) or Guanosine (G) at nucleotide 10434 of the 3′ NCR (Table 3). As with VEEV TRD, the passage history of JEV Nakayama prior to initiating these studies was not known. Passing JEV Nak in or between mammalian and mosquito cells selected for Lys at E 209 and NS4b 8 and A at nucleotide 10431 of the 3′ NCR. Passage in eggs or mice selected for Arg or G at these positions, respectively. Val was selected at NS5 405 and U was selected at nucleotide 10431 of the 3′ NCR regardless of the passage condition.

JEV MB and JEV Egg have one and two additional amino acid changes, respectively, in comparison to JEV Nak that were confined to the E protein (Table 3). None of these changes was conserved between JEV MB and JEV Egg. Genetic changes observed in JEV Egg were introduced after 5 or more passes and became predominant in the majority population sometime before 20 passes (data not shown).

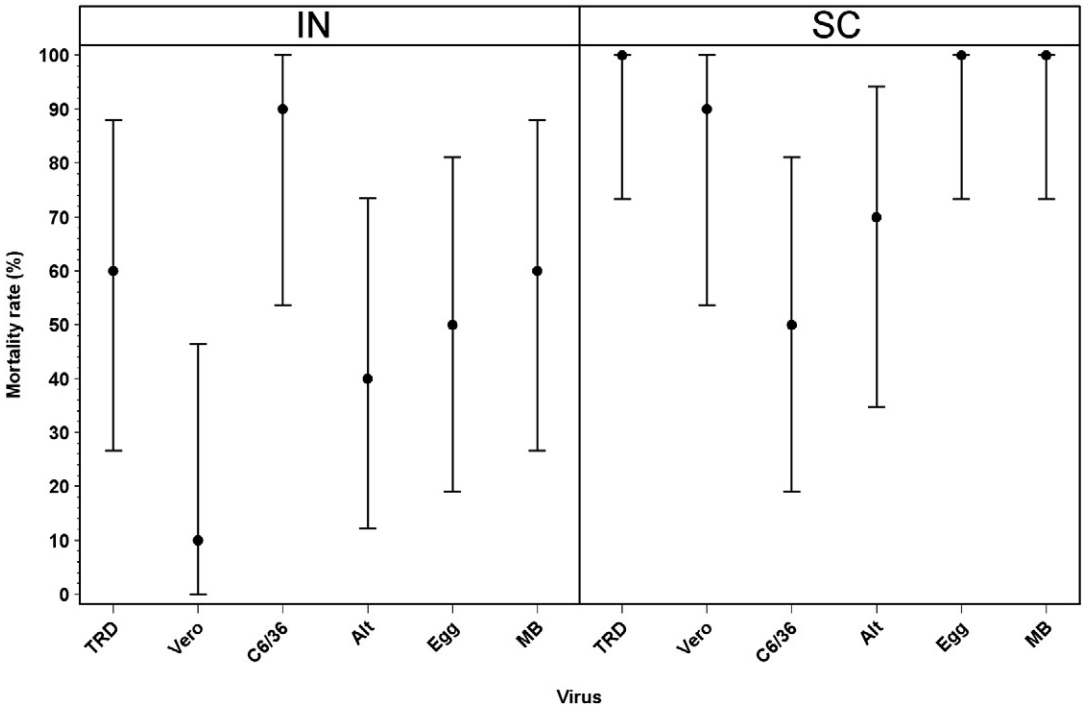

Fig. 1. Percent mortality and 95% confidence bounds for weanling CD-1 mice challenged by the IN or SC routes with 1×10^4 pfu VEEV TRD (parent), VEEV Vero, VEEV C6/36, VEEV Alt, VEEV Egg or VEEV MB.

Passing JEV Nak in tissue culture cells resulted in numerous additional genetic changes that were distributed throughout the viral genome (Table 3). JEV Alt had 21 nucleotide or amino acid changes in comparison to JEV Nak. JEV Vero had 22 changes in total and JEV C6/36 had 21. Of these nucleotide and amino acid changes, 17 were conserved between JEV Alt, JEV Vero and JEV C6/36. Conserved genetic changes were observed in the 5′ and 3′ NCR, and in the capsid (C), E, NS1, 2K, and NS4b proteins (Table 3). Each of these conserved changes was selected within the first 5 passes of JEV Alt, JEV Vero and JEV C6/36 (Table 4).

Effect of passage on the virulence of JEV

IN and SC exposure of weanling CD-1 mice with JEV Nak resulted in 80% and 90% mortality, respectively (Fig. 3). Challenging mice with

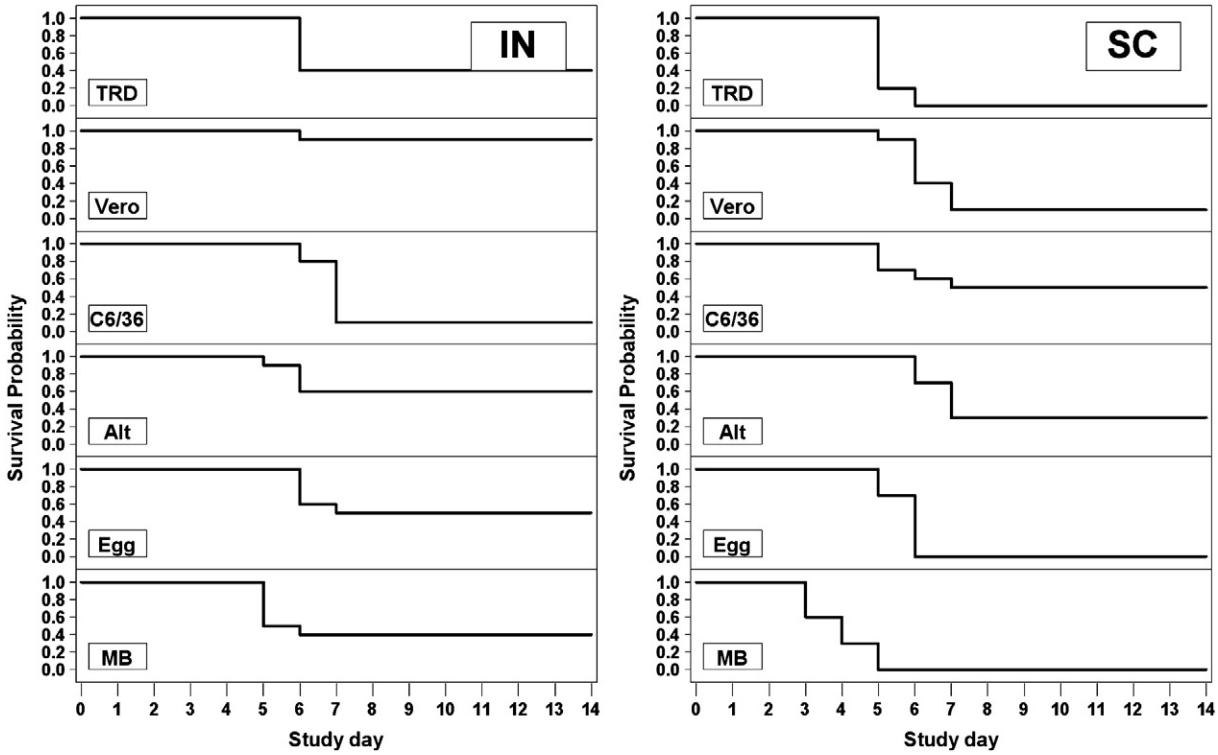

Fig. 2. Kaplan–Meier survival curves for weanling CD-1 mice challenged with 1×10^4 pfu VEEV TRD (parent), VEEV Vero, VEEV C6/36, VEEV Alt, VEEV Egg or VEEV MB. (A) = IN administration. (B) = SC administration.

Table 3
Nucleotide and amino acid changes in JEV following passage.

Region[a]	Position[b]	Virus Stock					
		JEV Nak	JEV Vero	JEV C6/36	JEV Alt	JEV Egg	JEV MB
5′ NCR	nt14	C	U	U	U	–	–
	nt49	U	C	C	C	–	–
C	aa120	Ile	Val	Val	Val	–	–
PreM	aa110	Ala	Val	–	–	–	–
E	aa82	Lys	–	Glu	–	–	–
	aa83	Lys	Glu	Lys/Glu	Glu	–	–
	aa124	Lys	–	–	Lys/Glu	–	–
	aa176	Thr	Ile	Ile	Ile	–	–
	aa191	Glu	–	–	Ala	–	–
	aa209	Lys/Arg[c]	Lys	Lys	Lys	Arg	Arg
	aa228	Pro	–	–	–	Ala	–
	aa232	Ala	–	–	–	Val	–
	aa275	Asn	–	–	–	–	Asp
	aa290	Arg	Lys	Lys	Lys	–	–
	aa306	Glu	–	Glu/Gly	–	–	–
	aa418	Val	Ala	Ala	Ala	–	–
NS1	aa8	Val	Ile	Ile	Ile	–	–
NS2a	–	–	–	–	–	–	–
NS2b	–	–	–	–	–	–	–
NS3	aa249	Gln	–	Gln/Arg	–	–	–
	aa443	Ile	Leu	–	–	–	–
	aa518	Leu	Phe	–	Phe	–	–
NS4a	–	–	–	–	–	–	–
2K	aa20	Met	Val	Val	Val	–	–
NS4b	aa8	Lys/Arg	Lys	Lys	Lys	Arg	Arg
	aa110	Val	Phe	Phe	Phe	–	–
NS5	aa405	Ile/Val	Val	Val	Val	Val	Val
	aa643	Arg	Lys	–	–	–	–
3′ NCR	nt10408	A	–	–	–	A/G	–
	nt10410	G	A	A	A	–	–
	nt10431	C/U	U	U	U	U	U
	nt10434	A/G	A	A	A	G	G
	nt10448	G	A	A	A	–	–
	nt10554	G	A	A	A	–	–

–, no change.
[a] Non-coding region (NCR) or viral protein.
[b] Nucleotide (nt) or amino acid (aa) position within the viral genome or within a given gene.
[c] Mixed population.

JEV Alt and JEV MB by the IN route had no significant effect on the percent mortality in comparison to JEV Nak and the survival probabilities were unchanged (Fig. 4). A significant decrease in the percent mortality and improved survival probabilities were observed in mice challenged IN with JEV Vero, JEV C6/36 and JEV Egg ($P=0.005$ or less). Following SC exposure JEV Vero, JEV Alt, JEV Egg, and JEV MB all demonstrated a decrease in percent mortality when compared to JEV Nak; however only VEEV C6/36 demonstrated a significant decrease ($P=0.001$). All passage stocks exhibited significantly improved survival probabilities when compared with JEV Nak following SC exposure ($P=0.019$ or less). The decrease in percent mortality following SC exposure of mice with JEV Vero, JEV C6/36, JEV Alt and JEV Egg, was confirmed in pass 5 stocks (data not shown). Because all of the conserved genetic changes observed in JEV were present in the pass 5 stocks the contribution of specific genetic changes on the attenuated phenotype could not be determined.

Discussion

These studies demonstrate conclusively that the consequence of eliminating alternate replication in vertebrate and invertebrate cells on the accumulation of genetic changes in VEEV and JEV is different and may be dependent on the method in which the virus is cultured. For VEEV, few genetic changes were observed during the course of these investigations, and as a result, the percent mortality in mice was not significantly altered. A Lys residue at E2 120 is a known tissue culture adaptation of VEEV and was previously identified as being one of two genetic changes responsible for the attenuation of the VEEV live vaccine strain TC-83(Kinney et al., 1993). Although the passage history of VEEV TRD prior to initiating these studies was unknown, the presence of both Thr and Lys at this position in the stock of VEEV TRD suggests that the virus had already been partially adapted to growth in tissue culture cells. Passing VEEV TRD in mouse brains and in eggs 20 times selected for the wild-type Thr at this position. The decreased survival probability observed with VEEV MB following SC exposure is consistent with what was observed previously with SINV (Lustig et al., 1992); but may simply represent a reversion of the virus to the wild-type phenotype as a result of the E2 120 amino acid change. An additional amino acid change in the E2 glycoprotein of VEEV MB was present and a similar phenotype change was not observed with VEEV Egg; thus additional studies are required to determine the contribution of these genetic changes to the phenotype of the virus. Passage of VEEV TRD on or between mammalian and mosquito tissue culture cells selected for Lys at E2 120 consistent with previous results on tissue culture adaptation of VEEV TRD (Kinney et al., 1993). Regardless of whether the virus was passed *in vitro* or *in vivo*, 10 to 20 passes of VEEV TRD were required for the amino acid change at E2 120 to become fixed in the population and additional genetic changes in the VEEV genome were equally slow to develop. Thus, the effect of different culture methods on the accumulation of genetic changes in VEEV TRD as observed in these studies is independent of the previous passage history.

Passing JEV Nak under the identical conditions resulted in numerous genetic changes that were distributed throughout the viral genome and in most cases; these genetic changes were introduced in 5 passes or less and remained fixed in the population for all subsequent passes. Consequently, all of the viruses generated following passage of JEV, including those passed in eggs and mice, demonstrated decreased mortality and improved survival probabilities in mice to different extents depending upon the route of inoculation. For each of the JEV stocks tested, attenuated virulence was observed within the first 5 passes (data not shown). Because of the number of genetic changes observed and the rapidity at which they were selected for JEV, the contribution of specific genetic changes on the virulence of the virus for mice could not be determined by challenging mice with earlier passes of the virus. Additionally, it has been demonstrated previously that genetic changes in the NCR and the nonstructural proteins of flaviviruses as well as the structural proteins can influence virus virulence; thus it is not possible to speculate on the role of individual genetic changes on the observed phenotype (Butrapet et al., 2000; Chambers et al., 2007; Durbin et al., 2001). Passing JEV in eggs and mice resulted in the fewest number of genetic changes although only two were conserved between the *in vitro* and *in vivo* passage stocks. These included a Val residue at NS5 405 and a U at nucleotide 10431 of the 3′ NCR. Passing JEV in or between Vero and C6/36 cells resulted in 11 nucleotide or amino acid changes that were conserved and likely represent tissue culture adaptations. In order to determine the contribution of each of these amino acid changes on the virus phenotype, each of the nucleotide and amino acid changes observed in this study could be investigated individually or in combination using currently available infectious clones developed in other labs.

The reasons for the differences observed between VEEV and JEV in these studies was not evaluated and since only one strain of each was used, it is not possible to know if these observations would apply equally to other strains of VEEV and JEV or other viruses in the alphavirus and flavivirus genera. Despite their many similarities, the organization of the VEEV and JEV genome is dissimilar as is the manner in which the viral RNA is translated and transcribed [for reviews see (Fernandez-Garcia et al., 2009; Jose et al., 2009)]. The genome of JEV encodes a single long polyprotein that is translated to produce structural and nonstructural proteins simultaneously. The structural proteins of JEV are produced first because of their location

Table 4
Accumulation of amino acid changes in JEV following passage in tissue culture cells.

Region[a]	Position[b]	Virus stock								
		JEV Nak	JEV Vero P5	JEV Vero P10	JEV Vero P15	JEV Vero P20	JEV Vero P25	JEV Vero P30	JEV Vero P35	JEV Vero P40
C	aa120	Ile	Val	Val	Val	Val	Val	Val	Val	Val
PreM	aa110	Ala	–	–	–	–	–	Ala/Val	Ala/Val	Val
E	aa83	Lys	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu
	aa176	Thr	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile
	aa209	Lys/Arg	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
	aa290	Arg	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
	aa418	Val	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala
NS1	aa8	Val	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile
NS2a	–	–	–	–	–	–	–	–	–	–
NS2b	–	–	–	–	–	–	–	–	–	–
NS3	aa443	Ile	–	–	–	Leu	Leu	Leu	Leu	Leu
	aa518	Leu	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe
NS4a	–	–	–	–	–	–	–	–	–	–
2K	aa209	Met	Val	Val	Val	Val	Val	Val	Val	Val
NS4b	aa8	Lys/Arg	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
	aa110	Val	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe
NS5	aa405	Ile/Val	Val	Val	Val	Val	Val	Val	Val	Val
	aa643	Arg	–	–	–	–	–	–	–	Lys

Region[a]	Position[b]	Virus Stock								
		JEV Nak	JEV C6/36 P5	JEV C6/36 P10	JEV C6/36 P15	JEV C6/36 P20	JEV C6/36 P25	JEV C6/36 P30	JEV C6/36 P35	JEV C6/36 P40
C	aa120	Ile	Val	Val	Val	Val	Val	Val	Val	Val
PreM	–	–	–	–	–	–	–	–	–	–
E	aa82	Lys	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu
	aa83	Lys	–	–	–	–	–	Lys/Glu	Lys/Glu	Lys/Glu
	aa176	Thr	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile
	aa209	Lys/Arg[c]	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
	aa290	Arg	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
	aa306	Glu	–	–	–	–	–	Glu/Gly	Glu/Gly	Glu/Gly
	aa418	Val	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala
NS1	aa8	Val	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile
NS2a	–	–	–	–	–	–	–	–	–	–
NS2b	–	–	–	–	–	–	–	–	–	–
NS3	aa249	Gln	–	–	–	–	–	Gln/Arg	Gln/Arg	Gln/Arg
	aa518	Leu	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe
NS4a	–	–	–	–	–	–	–	–	–	–
2K	aa209	Met	Val	Val	Val	Val	Val	Val	Val	Val
NS4b	aa8	Lys/Arg	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
NS5	aa405	Ile/Val	Val	Val	Val	Val	Val	Val	Val	Val

Region[a]	Position[b]	Virus stock								
		JEV Nak	JEV Alt P5	JEV Alt P10	JEV Alt P15	JEV Alt P20	JEV Alt P25	JEV Alt P30	JEV AltP35	JEV Alt P40
C	aa120	Ile	Val	Val	Val	Val	Val	Val	Val	Val
PreM	–	–	–	–	–	–	–	–	–	–
E	aa83	Lys	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu
	aa124	Lys	–	–	–	–	–	–	–	Lys/Glu
	aa176	Thr	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile
	aa191	Glu	–	–	–	–	–	–	–	Ala
	aa209	Lys/Arg†	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
	aa290	Arg	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
	aa418	Val	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala
NS1	aa8	Val	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile
NS2a	–	–	–	–	–	–	–	–	–	–
NS2b	–	–	–	–	–	–	–	–	–	–
NS3	aa518	Leu	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe
NS4a	–	–	–	–	–	–	–	–	–	–
2K	aa20	Met	Val	Val	Val	Val	Val	Val	Val	Val
NS4b	aa8	Lys/Arg	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
	aa110	Val	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe
NS5	aa405	Ile/Val	Val	Val	Val	Val	Val	Val	Val	Val

–, no change.
[a] Viral protein.
[b] Amino acid (aa) position within a given gene.
[c] Mixed population.

in the genome followed by the nonstructural proteins which function to replicate the viral RNA in a continuous cycle. For VEEV, the nonstructural protein genes are located in the 5′ two-thirds of the genome and are the first to be translated. The nonstructural proteins of VEEV serve to replicate the viral RNA as well as to produce a subgenomic RNA that encodes the structural proteins, which are consequently produced at later points during infection of a cell. Differences in protein expression and RNA replication affect the way in which VEEV and JEV viral titers accumulate in infected cells and animals (data not shown). These differences also may affect the accumulation of genetic changes in VEEV and JEV accounting for the disparity observed in these studies. Repeating the passage studies

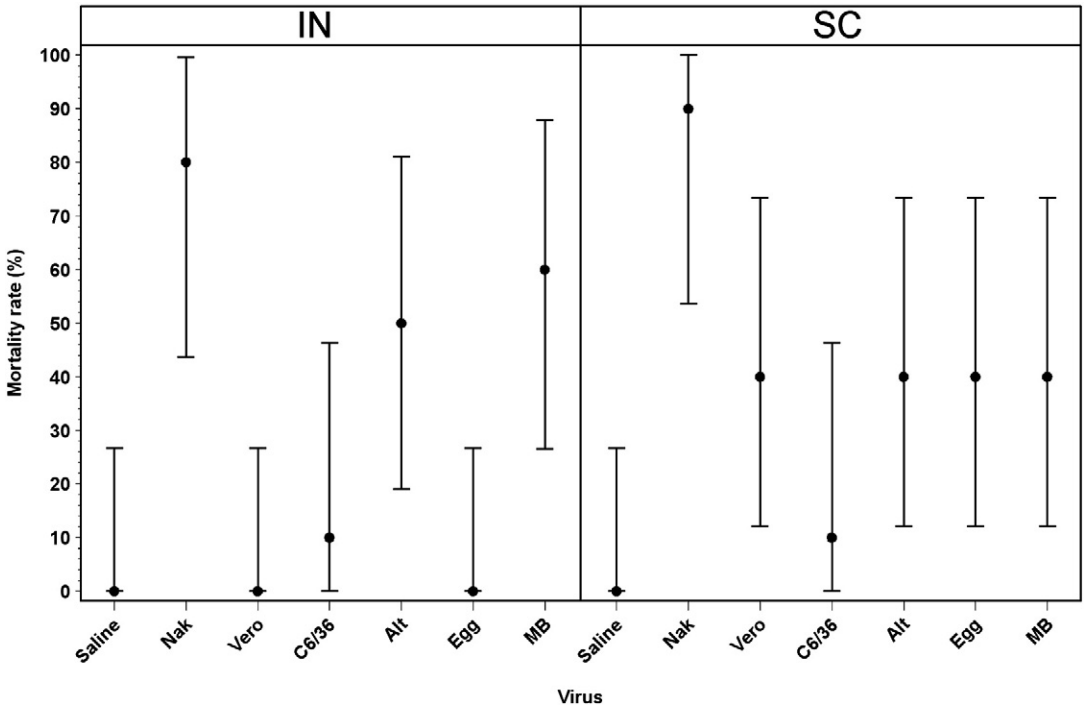

Fig. 3. Percent mortality and 95% confidence bounds for weanling CD-1 mice challenged by the IN or SC routes with 1×10^4 pfu JEV TRD (parent), JEV Vero, JEV C6/36, JEV Alt, JEV Egg or JEV MB.

with different strains of VEEV and JEV as well as different viruses in the alphavirus and flavivirus genera are required in order to address this hypothesis.

For VEEV, the synthesis of complementary minus-strand RNAs is believed to be down regulated during cellular infection to favor the generation of new genomic and subgenomic RNAs (Jose et al., 2009; Shirako and Strauss, 1990). Since minus-strand RNA serves as template for genomic RNA that is subsequently packaged into numerous viral particles, genetic changes introduced at this level would be expected to be present in a greater percentage of the viral population; whereas genetic changes introduced into the genomic RNA would only be propagated during subsequent rounds of

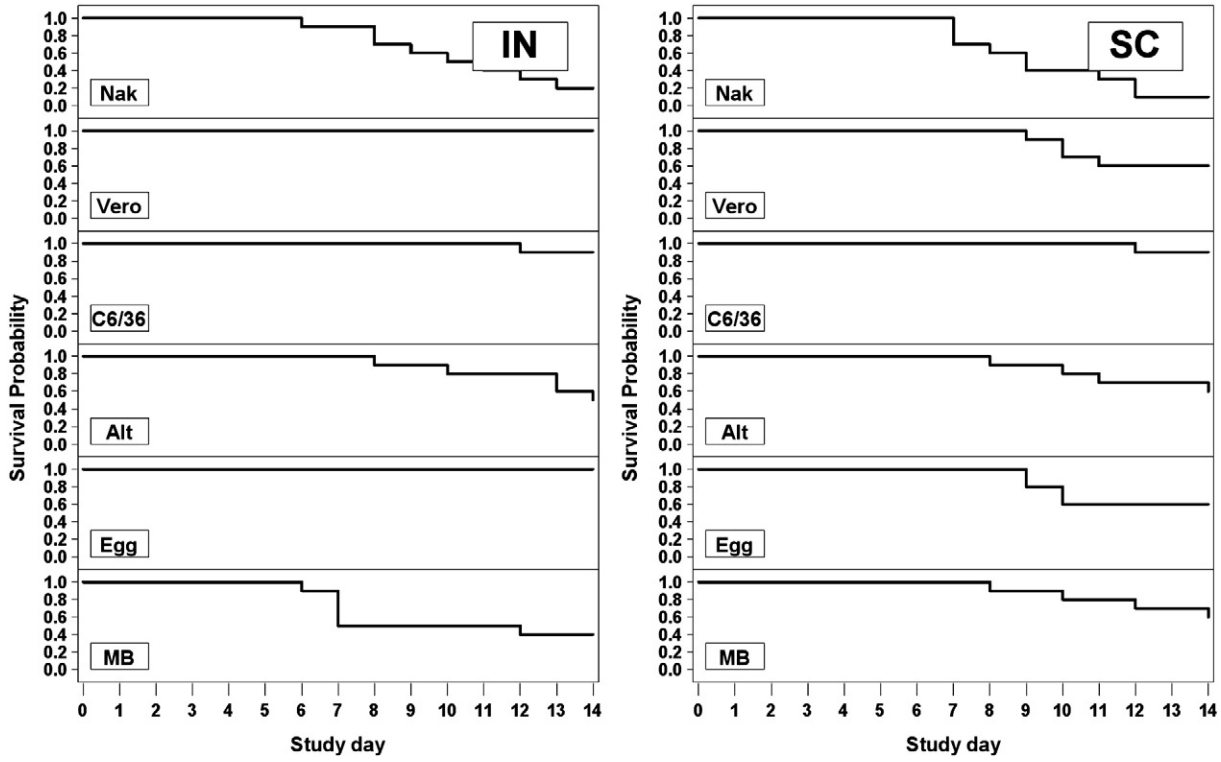

Fig. 4. Kaplan–Meier survival curves for weanling CD-1 mice challenged with 1×10^4 pfu JEV TRD (parent), JEV Vero, JEV C6/36, JEV Alt, JEV Egg or JEV MB. (A) = IN administration. (B) = SC administration.

infection. Consequently, down regulation of minus-strand RNA synthesis would be expected to reduce the probability of genetic changes being introduced into the majority population and might contribute to the genetic stability observed with VEEV in these studies. We can test the effects of down regulating minus-strand synthesis on the stability of the VEEV genome by artificially introducing genetic changes into an infectious clone of VEEV that would allow negative strand RNA synthesis to continue throughout cellular infection, as was demonstrated for SINV (Gorchakov et al., 2008), and repeat the passage experiments. If down regulation of minus-strand RNA synthesis does play a role in stability of VEEV, it would also be expected that the majority of genetic changes introduced during replication would be at the level of genomic RNA synthesis and, thus, the genetic population within a stock of VEEV would be more heterogeneous than reported here. Heterogeneity in the sequence of VEEV was not observed in these studies because the method used for sequencing only reports the most abundant genetic information within a population. The application of next generation sequencing technologies and bioinformatics tools to these questions would provide a great deal more data on the genetic population within a viral sample and, for VEEV, may aid in the identification of genetic signatures that would be associated with a given culture method.

Materials and methods

Cells and viruses

Vero, and C6/36 cells were cultured in Dulbecco's modified minimal essential media (DMEM) supplemented with 10% fetal bovine serum, nonessential amino acids, L-glutamine and Penicillin–Streptomycin–Neomycin (PSN) antibiotic mixture. Vero cells were maintained at 37 °C plus 5% CO_2. C6/36 cells were kept at 28 °C with 5% CO_2.

The VEEV Trinidad Donkey strain was obtained from Dr. Michael Parker at the USAMRIID in Frederick, MD; and the JEV Nakayama strain was obtained from Dr. Barbara Johnson at the CDC, Division of Vector-borne Infectious Diseases in Fort Collins, CO. Parental stocks of each virus were generated by infecting Vero cells in 175 cm^2 flasks and incubating at 37 °C and 5% CO_2 until approximately 80% cytopathic effect (CPE) was observed. Virus was collected in the culture supernatant, filtered using a 0.2 μm filter, aliquoted to 1.5 ml screw top cryovials and stored at −80 °C. The nucleic acid sequence of the complete viral genome was determined for each viral stock, along with viral titer after thawing from −80 °C storage as described below. Virus stocks were determined to be free of mycoplasma via polymerase chain reaction using a commercially available kit (Takara Bio Inc., Madison, WI).

Plaque assays were performed on Vero cells in six well plates by incubating cells with 0.2 ml of 10-fold serial dilutions of virus at 37 °C for 1 h. Monolayers were overlaid with 3 ml of Eagle's minimal essential medium (EMEM) containing 2% FBS, PSN, nonessential amino acids, L-glutamine, and 1% agarose. VEEV titrations were incubated for approximately 24 h before addition of 2 ml of secondary overlay (EMEM contained neutral red at 0.015%). JEV titrations received the secondary overlay at approximately 72 h post infection. Plaques were counted on sequential days up to 3 days after addition of the secondary overlay.

Mice

For newborn CD-1 mice, pregnant dams were ordered from Charles River Laboratories (Portage, MI) at 13 days gestation and housed individually in conventional animal facilities until delivery. For weanling CD-1 mice, 2 week old litters were purchased from Charles River Laboratories and housed with their dams for one week in conventional animal facilities before being separated into micro-isolator cages and transferred to the animal biosafety level 3 (BSL-3) facility. Food and water were provided *ad libitum* to all mice. This research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and in adherence to principles stated in the Guide for the Care and Use of Laboratory Animals (Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, NIH Publication No. 86-23, Revised 1996, NRC Publication, 1996 edition). All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Midwest Research Institute and the Army Animal Care and Use Review Office prior to the initiation of work. All work was performed in facilities certified by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

Tissue culture passage

Vero or C6/36 cells in 25 cm^2 flasks were infected with VEEV or JEV at an MOI of 0.1 in 2 ml of media. After 1 h the virus was removed, the cells were washed 2 times with 3 ml of PBS, and 8 ml of media was added back to the flasks. C6/36 cells were incubated at 28 °C with 5% CO_2 for 48 (VEEV) or 72 (JEV) h when peak titers were expected to be achieved in preliminary growth curve studies (data not shown). Vero cells were incubated at 28 °C with 5% CO_2 for 24 (VEEV) or 72 (JEV) h. The virus was collected from the culture supernatant, filtered through a 0.2 μm filter and aliquoted to cryovials for storage at −80 °C. Viral titers for all frozen stocks were determined by plaque assay on Vero cells following a single thaw on ice. All passes were performed by infecting the appropriate cells in culture at an MOI of 0.1 using virus of known titer that was thawed only once. After 22 passes, Vero cells infected with JEV were incubated for 48 h before the culture supernatant was collected due to changes in the accumulation of viral titers. The final pass (pass 40) was performed in 75 cm^2 flasks containing 20 ml of media. For vaccine strains of VEEV (TC-83) and JEV (SA-14-14-2) more than 80 to 100 passes, respectively, were required to select for an attenuated virus phenotype (Kinney et al., 1993; Yu, 2010); however, significant changes were observed in as few as 6 passes in preliminary studies conducted in our labs. We chose 40 passes for this study as a preliminary endpoint that could be extended depending on the results.

Mouse brain passage

Groups of 10 newborn CD-1 mice were inoculated ic with 1×10^3 pfu of VEEV or JEV in 0.020 ml. Pups were maintained with their dams for a period of approximately 24 (VEEV) to 48 (JEV) h post-inoculation before the brains were collected and pooled. The incubation times correspond to when peak virus titers were expected and were determined in preliminary time course experiments (data not shown). Viral stocks were prepared by transferring the pooled brains to a tissue homogenizer and adding sufficient volume of PBS to achieve a final concentration of 10% (weight/volume). Following homogenization, the stocks were filtered through a 0.22 μm filter, aliquoted to cryovials and stored at −80 °C. Viral titers were determined for each frozen stock via plaque assay on Vero cells following a single thaw. Subsequent passes were performed by inoculating new groups of newborn CD-1 mice as described above with VEEV MB or JEV MB stocks that had been thawed one time. The final pass was performed using 25 newborn pups. For SINV, between 15 and 22 passes were required to select for a neurovirulent and neuroinvasive phenotype (Lustig et al., 1992); therefore, 20 passes were used in these studies as a preliminary endpoint that would minimize the overall number of mice that would be required.

Egg culture passage

Groups of 3, eight day old SPF chicken eggs were inoculated with 5×10^3 pfu of VEEV or JEV at the yolk sack and incubated for a period of 24 (VEEV) to 48 h (JEV) at 39 °C. The incubation times and the concentration of virus in specific egg tissues and fluids were determined in preliminary time course studies (data not shown). Infected eggs were kept at 4 °C for a minimum of 4 h before the embryo's were collected, pooled and homogenized to a final concentration of 10% (weight/volume) in PBS. Stocks of virus were prepared by filtering, aliquoting to cryovials, and storing at −80 °C. A 0.45 μm filter was used for these stocks because the 0.22 μm filter became clogged by the homogenized tissues and minimized the volume recovered following harvest. Virus titers from stocks that were thawed one time were determined by plaque assay on Vero cells. Subsequent passes were performed as described above using virus stocks that had been thawed one time. A total of 6 eggs were used for the final pass (pass 20). The endpoint for egg passage studies was chosen to correspond with the mouse brain passage study and minimize the number of eggs that would be required.

Sequencing

For genomic sequencing, viral RNA was extracted from stocks using the QIAamp viral RNA minikit (Qiagen) according to the manufacturer's protocol. RNA was eluted in 50 μl of RNase free H_20 and 10% was tested in an *in vitro* assay for sterility before removing from the lab.

A fraction of the extracted RNA was A-tailed using Poly-A Polymerase according to manufacturer protocol (Ambion). Adding additional A nucleotides to the VEEV RNA significantly improved the recovery of cDNA. Full-length genomic cDNA were then generated using the MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre) according to manufacturer protocol. Amplicons corresponding to overlapping segments of the viral genomes were amplified from the cDNAs using virus specific primer sets (Tables 5 and 6) according to the following thermal protocol: 95.0 for 3 min, 35 cycles of 95.0 for 30 s, 64.0 for 30 s, 72.0 for 2 min, followed by a 2-min extension at 72.0.

RACE-ready cDNA was generated using a polyA (3′ end) or specific (5′ RACE 1) primer according to manufacturer protocol for 3′ (Clontech, Mountain View, CA) and 5′ (Invitrogen) 1st strand synthesis. 5′ cDNA was then terminal deoxynucleotidyl transferase tailed prior to amplification according to manufacturer's protocol. RACE-prepared cDNA was amplified using virus specific primers (Tables 5 and 6) for both the 3′ (3′ RACE) and 5′ (5′ RACE 2) ends according to the following thermal protocol: 94.0 for 2 min, 40 cycles of 94.0 for 30 s, 68.0 for 30 s, 72.0 for 1 min followed by a 2-min extension.

Table 5
Primer sets for amplification of the VEEV genome.

Fragment	Primer ID	Sequence (5′–3′)
1	VEEV_00509F	GGTTGACGGACCGACAAGTCTC
	VEEV_02145R	AGGCGAATTCATGGAAGGGAGG
2	VEEV_01978F	GAGGAGCGCTGAACACTGATG
	VEEV_03637R	AGCCGAGCTCTGAAGGTAGC
3	VEEV_03467F	GCCTCATGCTTTAGTCCTCCACC
	VEEV_05082R	CCTCTGTGGATTGGTTCTCTGC
4	VEEV_04946F	GCAGAAGATCCAATGCTCCCAG
	VEEV_06642R	CAGCCTGGATCACCTGTACC
5	VEEV_06533F	GCAGGACATACCAATGGACAGG
	VEEV_08180R	CTGCTCCATGATGCCAGCTG
6	VEEV_08018F	GTGGAAGGCAAGATCGACAACG
	VEEV_09665R	TGGTGGACATAGGGTATCTGTGG
7	VEEV_09473F	CCTCACTACACGCACGAGCTC
	VEEV_10874R	CTGACACCCTGGTGAACAAGG
5′ RACE 1	VEEV_RACE_00831R	CAGACGGCAGGTGCCAGCTCCTCAGTAAGTCCCTC
5′ RACE 2	VEEV_RACE_00732R	TTCTAAGAATGGACATCCCTCTACGTGACCGCTCC
3′ RACE	VEEV_RACE_10660F	GCTGCAGAGACCCAAAGCAGGAGCGATCCACGTG

Table 6
Primer sets for amplification of the JEV genome.

Fragment	Primer ID	Sequence (5′–3′)
1	JEV_00420F	GAAGGCTCAATCATGTGGCTCG
	JEV_01871R	GCCTTTCAGAGCCAGTTTGTCC
2	JEV_01712F	GCACGCCACAAAACAGTCC
	JEV_03418R	CGACAGCACCAGTCAGTGATC
3	JEV_03232F	GCAAGCACAATCGGAGGGAAG
	JEV_04969R	CCGAAGGGAGTCCGAAACAC
4	JEV_04744F	TCCACACACTATGGCACACAAC
	JEV_06284R	GGTGTACTGAATGCCATTGGACG
5	JEV_06173F	CGAATACCGTCTCAGAGGTGAAG
	JEV_07951R	GCTGCGTAGTAGCTCCATCC
6	JEV_07760F	GAGAGAGGCCATAATCGAGGTGG
	JEV_09441R	CTTCTGCTGCAGGTCTCATGAC
7	JEV_09185F	GAATTCAGGAGGTGGAGTGGAAGG
	JEV_10631R	TTTGTGGCCTGACGTTGGTC
5′ RACE 1	JEV_RACE_00740R	CTTGGAATGCCTGGTCCGCGTGCACCGTCC
5′ RACE 2	JEV_RACE_00601R SNP1	CCGACGTCGATTGCCCGGACCCAACATCTG
3′ RACE	JEV_RACE_10228F SNP1	GGTGTGGCAGCCTTATCGGAACGCGATCC

Primers were removed using the QIAquick PCR Purification Kit and samples were eluted in 50 μl of molecular grade water. Each sample was sequenced in both directions using BigDye Terminator v3.1 (Applied Biosystems, Carlsbad, CA) and analyzed on an ABI 3130 capillary sequencer. Sequences were manually trimmed and evaluated for quality. Sequence alignments were generated using Lasergene software (DNASTAR).

Virulence assay

Groups of ten weanling CD-1 mice were challenged with 1×10^4 pfu of VEEV or JEV virus stocks by the IN or SC routes. IN doses were administered in 0.020 ml total volume using a pipette to directly apply 0.010 ml inoculums to each nostril of an animal anesthetized with isofluorane. Administration of the dose was verified by visual inspection of the dose site and anesthetized animals were allowed to recover completely before being returned to their cages. SC inoculations were performed using a 25 gauge needle to administer 0.200 ml inoculums to a fold of skin at the back of the neck. Following return to their cages and racks, mice were observed for a period of up to 14 days for clinical signs of disease or death. Moribund animals were euthanized, as there is no evidence for recovery from clinical disease with EEEV or JEV, and the day of euthanasia was recorded as the day of death.

The percent mortality and 95% confidence bounds were calculated for each of the viruses by each of the routes. The percent mortality represents the total number of mice that died as a result of virus challenge in comparison to the total number of animals that were challenged. The Fisher's Exact Test was used to compare the percent mortality for each virus to the parent virus in order to identify significant differences. When comparing the virulence of different virus stocks, particularly when comparing a single dose level, the rate at which animals succumb to infection is also important and can be determined by calculating the survival probabilities which focus on the time to death as well as the likelihood of death. In this case, Kaplan–Meier Survival Curves are used to assess the percent of mice that survive challenge over time for each virus and route. To determine if the survival curves are equivalent between each virus and the parent the Log-rank test is used.

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